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Title: SELECTED USES OF ENZYMES WITH CRITICAL FLUIDS

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Selected uses of enzymes with critical fluids in analytical chemistry

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Abstract

The use of enzymes coupled with supercritical fluid-based analytical techniques, such as supercritical fluid extraction (SFE), provides a safer environment platform for the analytical chemist and reduces the use of organic solvents. Incorporation of such

techniques not only reduces the use of solvent in analytical laboratories, but can lead to overall method simplification and time savings. In this review some of the fundamental aspects of using enzymes in the presence of supercritical fluid media are discussed, particularly the influence of extraction (reaction) pressure, temperature, and water content of the extracting fluid and/or sample matrix. Screening of optimal conditions for conducting reactions in the presence of SF media (Supercritical Fluid Reaction, SFR) can be readily accomplished on automated serial or parallel SFE instrumentation, including selection of the proper enzyme. Numerous examples are cited, many based on lipase-initiated conversions of lipid substrates, to form analytical useful derivatives for gas chromatography (GC), high performance liquid chromatography (HPLC), or supercritical fluid chromatography (SFC) analysis. In certain cases, enzymatic-aided processing of samples can permit the coupling of the extraction, sample preparation, and final analysis steps. The derived methods/techniques find application in nutritional food analysis, assaying of industrial products, and the micro- analysis of specific samples.

Introduction

Enzymes are highly specific catalysts that can be used in reactions to promote sample clean-up, separation and derivatization in a wide range of analytical applications. Several types of enzymes, e.g. lipases, esterases, proteases, amylases and oxidases, are bio-catalytically active and highly stable in organic solvents (1-5). This is particularly useful for applications where the reactants are lipophilic and of limited solubility in aqueous solvents. In addition, the specificity of the enzymes can be altered when the reaction media is changed from aqueous to non-aqueous. For example, lipases normally catalyze the hydrolysis of triacylglycerols to free fatty acids in aqueous solution, but they catalyze the formation of esters from alcohols or carboxylic acids in organic solvents or supercritical fluids (SFs) (6-8). The activity and stability of enzymes in SFs was first demonstrated using such enzymes as polyphenol oxidase (9), alkaline phosphatase (10), and lipase (11). Since then, there have been many published investigations considering both the activity (12-16) and the stability (17-22) of enzymes in SFs.

There are many additional advantages of performing enzymatically-catalyzed reactions under supercritical conditions. There is a beneficial environmental aspect, i.e. the use of a SF such as supercritical carbon dioxide (SC-CO_2) instead of organic solvents and enzymes as catalysts to do "green chemistry". In addition, there are several unique features of using SFs as a reaction medium instead of organic solvents (8). These include: (i) faster reaction kinetics; (ii) control of the solubility of the reactants; (iii) conducting reactions in one homogeneous phase; and (iv) easy separation of the solvent from the reaction mixture and resultant analytes. Furthermore, SFs usually permit

reactions to be run under mild conditions, moderate temperatures, with the exclusion of oxygen and light.

The SF-based instrumentation to achieve this goal can be automated, which increases sample throughput. Supercritical carbon dioxide is the most commonly utilized SF, due to its physical properties: (i) its low critical temperature and pressure (31°C and 73 atm, respectively), (ii) its chemically inertness, (iii) its availability in high purity and relatively low cost, and (iv) its low toxicity and flammability. However, other fluids can be used as well, as demonstrated with ethylene (23), near-critical propane (23, 24), fluoroform (23-25), ethane (23, 24) and sulfur hexafluoride (20, 23, 24).

There are several excellent published review papers addressing the basic phase equilibrium and kinetics of supercritical fluid reactions (SFR) (8, 26-35). A recent review by Carrapiso and Garcia (36) summarizes lipid extractions using SFE, microwave assisted extraction, as well as *in-situ* derivatization reactions using chemical catalysts in organic solvents. However, to the best of our knowledge, a review focused on the analytical use and applications in SFE/SFR have not been reported in the literature.

In this review, the advantages of using enzymes in super- and sub-critical fluids are discussed from an analytical perspective. Particularly, the applicability of using different lipases is treated, with a special emphasis on the lipase from *Candida antarctica* type B (Novozyme 435), because of its demonstrated compatibility with SFs. Important experimental parameters are discussed and ways to optimize their use are suggested. Pertinent applications are reviewed, including for example, the use of lipases for: (i) methyl ester formation in the determination of nutritional fat in meats (37); (ii) methyl ester formation in the determination of fatty acids in soapstocks (38) and plant

seeds (39, 40); (iii) esterification of tall oil for the determination of different compound classes (41); and (iv) hydrolysis/alcoholysis of fat and vitamin A esters for the determination of fat-soluble vitamins in foods (42). Future applications of interest in analytical chemistry are also discussed.

Enzymes of utility

Enzymes are classified according to the type of reaction they catalyze, and assigned a designation by the Enzyme Commission (EC), the first number which identifies their generic group (43), such as: 1. oxidoreductases; 2. transferases; 3. hydrolases; 4. lyases; 5. isomerases and 6. ligases. Several different types of enzymes have been used for reactions under supercritical conditions, especially hydrolases, which include lipases (EC 3.1.1.3) (11), esterases (EC 3.1.1.1) (3), proteases (EC 3.4.4.-) (44), amylases (EC 3.2.1.-) (45), alkaline phosphatase (EC 3.1.3.1) (10), β-D-galactosidase (EC 3.2.1.23) (46) and cellulase (EC 3.2.1.4) (47). Several oxidoreductases have also been used with SFs, including cholesterol oxidase (EC 1.1.3.6) (48), polyphenol oxidase (EC 1.10.3.1) (9), alcohol dehydrogenase (EC 1.1.1.1) (15) and horseradish peroxidase (EC 1.11.1.7) (49), as well as thermolysin (EC 3.4.24.27) (50). Figure 1 indicates the number of research papers dealing on the use of such enzymes with SFs.

Figure 1

More than ninety percent of the papers cited in Figure 1 are concerned with the use of lipases in SFs. Consequently, this class of enzyme will be discussed below in more detail.

Lipases

Lipases are the most commonly used enzymes under supercritical conditions, due to the solubility of reactants and products in SFs. In addition, their excellent stability, activity, and stereoselectivity in SFs have been demonstrated extensively (11, 12, 18, 21, 44, 51-59). Lipases can catalyze a plethora of reactions. There are several hundred commercially available lipases, which have been isolated from plants, animals or microorganism cultures. Several of these have been used as catalysts coupled with SFs. These specific lipases are listed in Table 1.

Table 1

Lipases are serine esterases with 3-D structures consisting of eight parallel β-sheets surrounded by α-helices (60). Most lipases have a lid covering the active site, which opens upon interacting with an oil/water interface for activity to be exhibited, i.e. interfacial activation. Hence, lipases require access to a lipid phase for them to catalyze reactions with long-chain acylglycerols most efficiently. The physiological role of lipases is digestive, but in non-aqueous media several other reaction paths are possible. These reactions can be classified as follow (31):

- Hydrolysis ($RCOOR' + H_2O \rightarrow RCOOH + R'OH$)
- Esterification ($RCOOH + R'OH \rightarrow RCOOR' + H_2O$)
- Interesterification ($RCOOR' + R''COOR''' \rightarrow RCOOR''' + R''COOR'$)
- Alcoholysis ($RCOOR' + R''OH \rightarrow RCOOR'' + R'OH$)
- Acidolysis ($RCOOR' + R''COOH \rightarrow R''COOR' + RCOOH$)
- Aminolysis ($RCOOR' + R''NH_2 \rightarrow RCONHR'' + R'OH$)

A simplified mechanism for such reactions listed above is shown in Figure 2 with triacylglycerol as model substrate.

Figure 2

Figure 2 reveals that the mechanism of the initial step (step 1) is identical for all the different reaction paths, in which a lipase-substrate complex is formed. The second step (step 2) differs, in which the attacking nucleophile (R4-OH) can be a water molecule (yielding hydrolysis) or an alcohol (yielding esterification). Interesterification, alcoholysis, acidolysis and aminolysis, reactions commonly termed transesterification reactions, initially undergo hydrolysis with water as attacking nucleophile, followed directly by esterification (61). For additional information concerning reaction mechanisms and chemistry of lipases, the reader is referred to several excellent books and review articles (60-62).

Esterases, like lipases, hydrolyze carboxylic acid esters without the need for a cofactor, and the general reaction mechanism is the same as the one illustrated in Figure 2. However, esterases primarily catalyze reactions with water-soluble esters or short-chain acylglycerols. They do not have a lid covering their active site; hence they do not require access to a lipid phase for interfacial activation as lipases do. Interestingly, the physiological role of most esterases is still unknown.

Immobilization

Enzymes used as catalysts in continuous SFE reactions are usually immobilized on a support material. Immobilization improves the stability and activity of the enzymes, and allows their reuse (63). The support material should be inert, porous to facilitate diffusion, mechanically strong, and loaded with enzyme at the level of at least one

monolayer. Moreover, it has been shown by Persson et al. (64) that a higher enzyme loading gives maximum activity at a lower water content than when used at a lower enzyme loading. Hydrophobic polymers such as Accurel MP1000® are commonly used, but silica gels (12, 65), ion exchange resins (12, 66), ceramics (66) and glass beads (12, 66-68) have also been used as support materials. Immobilization of the enzyme to the support material is in most cases performed by adsorption (12, 69), but covalent attachment to the support material by cross-linking is also utilized, frequently by incorporating glutaraldehyde (66-68). In addition, an enzyme can also be immobilized on silica gels by entrapment (70). Enzymes attached to hydrophobic membranes by adsorption or to nylon membranes by covalent linking have also been used in certain food processing applications (71).

Selectivity of lipases

The shape of the binding site differs among enzymes. Pleiss et al. (72) divided six lipases and two esterases into three groups depending on whether their binding sites were funnel-, crevice- or tunnel-like. For example, the binding sites of CALB and PCL are funnel-like, while sites associated with RML and ROL are crevice-like and CRL is tunnel-like (see Table 1 for enzyme abbreviations). These different shapes of binding sites affect the selectivity of the lipases towards the substrates. The selectivity can be stereoselective, regiospecific or lipid-class specific, as noted below.

The stereoselectivity of lipases has ubiquitously been used to resolve enantiomers of secondary alcohols (60), including applications where SC-CO_2 has been used as reaction medium for the production of enantiomerically-pure pharmaceutical compounds, such as menthol (65), ibuprofen (73), and 3-hydroxy esters (74, 75). The

enantioselectivity can be increased by modifying the shape of the substrate, for example by increasing the size of one of the substituent of the secondary alcohol. However, if the substrate is too large, or the binding site relatively small or narrow, there is a risk of steric inhibition at the binding site. This was shown to be a problem for the lipase-catalyzed hydrolysis and alcoholysis of α-tocopheryl acetate in SC-CO_2/ethanol (97:3, v/v), hexane/ethanol (87.5:12.5, v/v) and water-saturated di-isopropyl ether (76).

Many lipases are regiospecific, or 1,3-specific with respect to action on triacylglycerols. This means that they primarily catalyze reactions at the primary (outer) positions of a triacylglycerol. Some examples of 1,3-specific lipases are ROL, RML and PPL (see Table 1).

Several lipases are specific for various lipid-classes. For example, many lipases catalyze reactions with triacylglycerols much faster than with di- or mono-acylglycerols (77). In addition, some lipases favor long-chain fatty acids with respect to short-chain fatty acids.

Choosing the optimal enzyme

It is obvious that there are many commercially-available enzymes that differ in terms of their substrate specificity, stability, activity, and different types of selectivity or non-selectivity. In order to select a suitable enzyme for a specific analytical application a group of enzymes is screened in a set of experiments. For example, Frykman et al. (69) screened ten immobilized lipases for their ability to form methyl esters using analytical "inverse" packed-bed SFE, and compared their exhibited activity in SC-CO_2 with their hydrolytic activity in aqueous media. They found that the lipase activity and substrate specificity exhibited in the two solvent systems were completely different. It is for this

reason advisable to perform such screening experiments in the presence of the SF. Moreover, parameters that have a major impact on the enzyme activity and stability should also be varied during these screening experiments, in order to make a confident rejection of enzymes that exhibit a low activity.

A lipase that has found wide applicability is immobilized *Candida antarctica* Type B (commercialized by Novozymes A/S, Bagsvaerd, Denmark, and manufactured as Novozyme 435). This particular lipase has only a small lid configuration and shows only a minor dependence on interfacial activation (60). Moreover, it demonstrates a high enantioselectivity towards alcohols, but a low enantioselectivity towards carboxylic acids. Immobilized CALB (Novozyme 435) has broad substrate specificity, and works well as a catalyst in analytical continuous flow-SFE systems (37-40, 76, 78, 79), as discussed below.

Parameters of the SFE/SFR system

The parameters of the supercritical system should be optimized in order to achieve the highest possible activity and stability of the enzyme. A high catalytic activity is of high importance, since it directly affects the reaction rate and production of desired analytes. Important extraction/reaction parameters that influence enzyme activity are, temperature, pressure and water content of the SF and enzyme. The stability of the enzyme affects its long-term activity, particularly with respect to the on-set of enzyme denaturation. Parameters that primarily influence long-term enzyme stability are the water content, temperature, and any cosolvents used with the SF. In addition, the yield of the enzyme-catalyzed reaction can be affected by the flow rate of the SF.

Water content in the SFE/SFR system

The water content of the system is one of the most important parameters to consider, as it affects both the activity and stability of the enzyme in a complex manner. The effect of water on enzyme catalysis can be summarized as follows:

- A small amount of water close to the enzyme surface, typically a monolayer, is needed for the enzyme to be active (80). This water is called "bound water" since it is tightly associated with the enzyme, and assists the enzyme in maintaining its active conformation. It has been shown by Randolph et al. (81) and Dumont et al. (82) that "bone-dry" SC-CO_2 removes bound water, thereby reversibly deactivating the enzyme.
- Generally, the enzyme activity increases with the water content of the support material, because water increases the flexibility of the enzyme (6).
- Water affects the course of the lipase- or esterase-catalyzed reaction, since water promotes hydrolysis. Water is a by-product in esterification reactions, and is initially needed in transesterification reactions even though its net production is zero (61).
- Too much water on the support material leads to mass transfer limitations, since the water forms a hydrophilic barrier hindering contacting between the reactants in the SF and the active sites of the enzyme (51).
- Large amounts of water may cause protein unfolding due to rupture of disulfide bonds and hydrolysis of peptide bonds, resulting in irreversible denaturation of the enzyme (82).
- Enzyme denaturation may also occur due to the release of bound water from the enzyme during the depressurization step (83).

The water concentration of a reaction system is usually described by the water activity, a_w (1). The a_w describes the level of enzyme hydration and can vary between 0 (for a dry system) and unity (for pure water). If the system is at equilibrium, the a_w will be the same in all phases of the system, i.e. support material and in the SF for a two-phase SFE system. Different fluids or fluid/cosolvent mixtures will yield a different distribution of water on the support material, depending on their ability to dissolve water. Hence, the optimal initial water content of a fluid for a given reaction may vary widely between different fluids, but the optimal a_w should be kept constant. It is therefore of fundamental importance to optimize the a_w of the system, and to try and keep it as constant as possible during the extraction/reaction process, as adding water continuously to the SF if needed. Organic solvents and enzyme preparations in a closed atmosphere can be pre-equilibrated to a desired a_w using saturated salt solutions (84). In a supercritical system, the distribution of water between the SF and the support material can be determined experimentally using an aluminum oxide humidity sensor (85) or Karl-Fischer titration (12, 86).

The water distribution will depend on the properties of the SF (pressure, temperature, etc.) and the type of support material. Generally, enzyme denaturation (due to protein unfolding) is promoted by water condensing from the SF onto the support material. Hence, the water solubility in the SF (87) is the most important factor to consider. An equation for calculating this, taking account of the effect of temperature and pressure, has been proposed by Chrastil (88). It is notable that the water solubility is only 0.878 mM (i.e. 0.016 g/L) in n-hexane at 40°C and ambient pressure, while it is about hundred times higher in SC-CO_2 at pressures above 15 MPa (26). With ethanol present as cosolvent, the solubility of water in SC-CO_2 at 40°C and 13 MPa is 2.11 g/L (300 mM

ethanol) and 2.9 g/L (450 mM ethanol), compared to 1.51 g/L with no ethanol added (86).

Marty et al. (86) studied the effects of water content on the RML-catalyzed esterification reaction between oleic acid and ethanol in both SC-CO_2 and hexane. Water adsorption isotherms were determined between the SF and the enzyme support material using Karl-Fisher titration of the support material, as well as the ethanolic cosolvent, after a specific incubation time, and with slow depressurization of the SF. It was shown that a water content of the support material of ~10% (w/w) gave the highest activity (measured as the initial reaction rate), independent of operation conditions or reaction media. Several other researchers have likewise found "bell-shaped" curves for enzyme activity versus water content (51, 89-91). It was also demonstrated by Marty et al. (86) that the adsorption of water to the support material in the SF system was negatively affected by increasing the temperature, the pressure, or the ethanol concentration.

The effect of water content of the support material on the enzyme stability was investigated by Chulalaksananukul et al. (19) for the RML-catalyzed alcoholysis reaction between geraniol, and esters like propyl or ethyl acetate. In this case, the addition of water to the SF system caused a decrease in residual enzyme activity (i.e. enzyme stability) for each temperature investigated (40, 60, 80 and 100°C). For every increase in temperature a corresponding decrease in residual enzyme activity was also recorded. Hence, it can be concluded that RML undergoes thermal denaturation, and water accelerates the process. Several other excellent publications describe the effects of the water content on enzyme activity and stability under supercritical conditions (12, 51-53).

Temperature

The reaction temperature affects both the activity and stability of the enzyme. Increasing temperature increases diffusion rates, thereby resulting in faster reactions, since the reaction rate is in most cases limited by slow diffusion of larger molecules into the porous enzyme support material. However, high temperatures increase the risk of thermally denaturing the enzyme, which is accelerated when water is present. Hence, there is usually an optimal reaction temperature, which is dependent on type of enzyme, support material, immobilization technique and reaction medium. Habulin et al. (92) demonstrated that for immobilized RML in a solvent-free system at ambient pressure, an increase in activity occurred with temperature up to 50°C, and thereafter, enzyme activity decreased at higher temperatures due to thermal denaturation. However, Overmeyer et al. (18) showed excellent stability and activity for Novozyme 435 in dry SC-CO_2 at temperatures above 100°C. A similar trend was also found by Turner et al. (76) in lipase-catalyzed transformation of retinyl palmitate to retinol in analytical packed-bed SFE. These results are shown in Figure 3 and demonstrate the high temperature stability of CALB in SC-CO_2 containing 3 vol% of ethanol and 0.15 vol% of water. RML on the other hand, was found to gain activity when the temperature was increased from 40 to 60°C, but lost activity at 80°C.

Figure 3

Pressure

The reaction pressure primarily influences the enzyme activity, whereas the effect on enzyme stability is less pronounced. An increase in pressure of the SF normally enhances the conversion rate due to increased analyte solubility (67, 93), however, at

some point, the enzyme activity starts to decrease with increasing pressure (18, 94). This has been attributed to the lower mass transfer rates of reactants with increase in SC-CO_2 density (94). Moreover, higher enzyme activity at near-critical conditions compared to supercritical conditions in CO_2 at higher pressure has been reported (73, 92, 95). Such effects were explained by the lower solubility of water in CO_2 (leaving more water available for the enzyme) (92), faster diffusion rates (73), and enhanced electron-accepting power of the SC-CO_2 (95).

The effect of pressure on the enzyme stability is usually quite small, even though it has been suggested that enzyme denaturation might occur during the depressurisation step due to the fast release of CO_2 dissolved in the enzyme-bound water. This effect was studied for trypsins (83), penicillin amidase (83), and crude CRL (21), and it was found that enzymes containing disulphide bridges were more stable to depressurization. Turner et al. (76) demonstrated that increasing the pressure from 2500 to 5500 psi (172 to 379 bar, at 70°C) resulted in an improved extraction and CALB-catalyzed reaction of retinyl palmitate to retinol from milk powder, resulting in 100% retinol recovery at 5500 psi (379 bar). It was also shown that at higher pressure, 7000 psi (483 bar), the retinol conversion dramatically dropped to below 20%. Hence, it appears that enzyme-denaturation is occurring during the course of the reaction at high pressures.

Cosolvents

Cosolvents such as ethanol or methanol are usually added to the SF to enhance the solubility of the analytes in the fluid and/or to facilitate the desorption of the analytes from the sample matrix as well as from the enzyme support material. Such cosolvents also serve as reactants in the reaction. In a study by Randolph et al. (81), it was

demonstrated that the addition of cosolvents caused aggregation of cholesterol molecules, which increased their rate of oxidation to cholestenone when catalyzed by cholesterol oxidase. However, there are negative effects of using cosolvents. For example, the cosolvent may participate in side-reactions, thereby giving rise to undesirable by-products. In addition, ethanol has been shown to inhibit enzyme activity (51, 52, 86, 90, 96) and also to dehydrate the enzyme, since water is more soluble in SF containing ethanol (52, 86). It is therefore important that the type of modifier and its concentration in the SF be carefully optimized. The distribution of cosolvent between the SF and the support material is determined by the solubility of the cosolvent in the SF, which in turn is controlled by the temperature, pressure and water content of the SF.

Flow rate

The flow rate of the SF in continuous packed-bed systems has the role of transporting the analytes from the sample matrix, through the enzyme-bed and finally to the collection device. The flow rate also can accelerate mixing, which even at low flow rates removes the rate-limiting effect of external diffusion (i.e. the diffusion from the bulk to the surface of the enzyme), due to the high solute diffusivities in SFs. A higher flow rate leads to a shorter mean residence time of the substrates in the enzyme bed. Therefore in dynamic systems, the lowest flow rate possible is commonly applied in order to maximize the reaction of analytes as they travel through the enzyme bed. However, lower flow rates give longer extraction/reaction times, which could be prohibitive in high-throughput laboratories. Dumont et al. (82) has demonstrated that a higher CO_2 flow rate increased the conversion rate of myristic acid and ethanol to ethyl myristate, although the mean residence time of analytes in the enzyme bed decreased. This was explained by the enhanced dilution of the ethanol by SC-CO_2, and ethanol has

an inhibiting effect on the enzyme activity. This interpretation was supported by performing another experiment where the substrate concentrations were kept constant in the SC-CO_2 while increasing the flow rate, which then gave rise to exponentially decreasing conversion. Gunnlaugsdottir and Sivik (97) showed that a higher flow rate could be used to remove products and by-products from the reaction mixture, and thereby shift the reaction equilibrium toward synthesis, and demonstrated that the flow rate affects the selectivity of the process, i.e. a higher flow rate showed bias toward the more soluble components. Hence, changing the flow rate in a SFR system is a convenient way of improving the yield and purity the reaction product.

Experimental aspects

Solubility of substrates in a supercritical fluid (SF)

A primary experimental variable is the solubility of the reaction substrates in the SF, since the substrate concentration should be as high as possible in order to approach highest reaction rate (98). This dependence is given by the Michaelis-Menten equation:

$$v = (V_{max} C_{sub}) / (K_m + C_{sub})$$

where v (μmol min^{-1} $g^{-1}_{catalyst}$) is the observed reaction rate, C_{sub} (M) is the substrate concentration, V_{max} (μmol min^{-1} $g^{-1}_{catalyst}$) is the limiting reaction rate, and K_m (M) is the Michaelis constant. Yoon et al. (99) showed that the reaction rate of RML-catalyzed interesterification of triolein (for the production of 1,3-dibehenoyl-2-oleoyl-glycerol, so called BOB) was much higher with ethyl behenate (EB) than with behenic acid (BA). This is due to the much higher solubility of the former substrate than the latter in SC-CO_2. The solubility of solutes in SFs has been described by Chrastil (88) as a function

of density of the SF (at specific temperatures and pressures), the molecular weights of solute and solvent, and the solutes' solvation number in SC-CO_2. If the solubility of the substrates is still not sufficient in the pure SF, cosolvents can be added to enhance their solubility. Heo et al. (94) showed that when tert-butanol was added as a cosolvent to SC-CO_2, it increased the solubility of methyl-β-D-fructofuranoside in SC-CO_2, enabling a high yield for the CALB-catalyzed acidolysis reaction with caprylic acid. However, as discussed previously, cosolvents may have negative effects on the enzyme, including competitive inhibition, steric hindrance, denaturation, and dehydration.

For example, Jackson and King (100) found an optimal methanol flow rate of 5 μL/min for the continuous CALB-catalyzed methanolysis of triacylglycerols from soy flakes. Lower flow rates were found to give a slower reaction rate and thereby a lower yield of fatty acid methyl esters (FAMEs), while higher flow rates inhibited the activity of the enzyme. If the substrates are highly polar, two alternative approaches have been suggested by Castillo et al. (101), in which the substrates were either adsorbed on silica gel or transformed to phenylboronic acid complexes. Using this approach, the solubility of glycerol and D-fructose in SC-CO_2 were significantly improved, enabling RML-catalyzed esterification to occur with oleic acid to produce mono- di- and triolein and fructose esters.

Effects of substrates and products on the enzyme activity

The size of the substrate can affect the reaction rate, as it is related to the fit of the substrate in the active site of the enzyme. Chulalaksananukul et al. (19) demonstrated that propyl acetate had the optimal ester chain length for the RML-catalyzed alcoholysis of geraniol to geranyl acetate in SC-CO_2. Esters of shorter chain length (methyl- and

ethyl acetate) as well as esters of longer chain length (butyl-, pentyl- and octyl acetate) gave rise to lower enzyme activity.

Both substrates and products can affect the enzyme activity by accumulating near the enzyme surface, and thereby sterically hinder new substrates from reaching the active sites(102). This problem can be overcome by assuring the reaction products and by-products are removed, for example by employing a semi-continuous or continuous flow reactor.

Types of reactor modules

Several different types of reactor modules have been used for enzymatically-catalyzed reactions in SFs, including: (i) batch reactor (23, 51); (ii) recirculating batch reactor (75, 86); (iii) extractive reactor (97, 103); (iv) semi-continuous reactor (68); (v) continuous reactor (104, 105); and (vi) automated analytical SFE/SFR system (37, 38, 42). These different reactor types are schematically shown in Figure 4.

Figure 4

In batch reactors and extractive reactors (Figure 4A and C) the impact of external diffusion limitations are greater than with recirculating or continuous packed-bed systems. Bernard and Barth (106) demonstrated the importance of stirring to avoid this external diffusion limitation. In packed-bed systems (Figure 4B,D-F) the flow of SF provides convection forces, leading to short diffusion distances through the boundary layer that exists between the bulk fluid and the enzyme. In addition, mass transfer limitations can be minimized by operating at elevated temperature and higher substrate concentration (30, 98). Such internal mass transfer limitations occur in both batch and continuous systems, due to slow diffusion inside the porous enzyme material. This

effect can be minimized by using smaller support particle sizes, which should yield faster reaction kinetics (106).

A major disadvantage with batch systems (Figure 4A-B) is the accumulation of products and by-products in the system, which slows down the reaction at some point due to their attainment of equilibrium in enclosed system. Adschiri et al. (107) demonstrated that extractive reaction gave a higher interesterification yield between tricaprylin and methyl oleate than when the reaction was conducted in the batch mode. This was due to continuous removal of the by-product, methyl caprylate, which shifted the equilibrium forward.

The use of enzymes in packed bed

It is not advisable to use crude enzymes in continuous packed-bed systems, since the high operating pressure will compress the fine enzyme particles into a compact plug, leading to a large pressure drop over the reactor column. Instead, enzymes immobilized on a suitable material, such as inert polymeric hydrophobic material (e.g. Accurel EP100®) or beads of glass or ceramics, should be employed. The amount of immobilized enzyme is not as important as the total length of the enzyme-bed, since this determines the residence time of the substrates in the reactor. The object in analytical work is to achieve quantitative yields in the reaction, which strongly depends on the residence time. A lower flow rate of the SF or, alternatively, of the substrates (if the substrate is continuously fed into the system), also results in a longer residence time, and enhanced conversion.

There are two options for facilitating an enzyme bed: in the extraction cell, or in a separate column. The enzyme bed is placed after the sample (or substrates) with respect

to the flow of the SF. Using the enzyme bed in the extraction cell, as opposed to having it in a separate column has the advantage that the reaction and extraction conditions are identical and that the average distance between the analytes in the sample and the enzyme bed is short. However, it is easier to handle and wash immobilized enzymes for reuse if they are packed in a separate column.

Immobilized enzymes can be washed and reused, which lowers the cost of the analytical process. It has been shown that immobilized CALB (Novozyme 435) can be recycled at least 25 times without decreasing its activity, when used during continuous analytical SFE with SC-CO_2, at 172 atm, 50°C and 1% of methanol as co-solvent (37). It is also advantageous to wash the fresh enzyme preparation (newly immobilized or purchased), in order to reduce initial contamination. Turner et al. (76) discovered that both commercially-obtained and laboratory-made immobilized lipase-preparations contained lipid components, which interfered with the chromatographic analysis of fat-soluble vitamins. It was shown that 30 minutes of treatment with SC-CO_2, using the same conditions as those applied during the enzymatic reaction, was sufficient to obtain clean enzymes with high activity. Giessauf and Gamse (13) also found that significant amounts of free fatty acids in PPL could be removed with SC-CO_2. Furthermore, Bauer et al. (108) demonstrated that treatment of crude esterase from *Bulkholderia gladioli* with SC-CO_2 resulted in 121% enzyme activity compared to 102% (nearly constant activity) for the already purified enzyme. In the same study (108), it was also demonstrated that the SC-CO_2 treated enzyme has significantly higher long-term stability in SC-CO_2 at 150 bar (2176 psi) and 75°C than crude *Bulkholderia gladioli* esterase.

Sample pre-treatment

If an automated analytical SFE system is used for subsequent extraction of a sample and enzymatic reaction of the extracted components, the sample pre-treatment can be of major importance. The water content of the sample is significant, since a large portion of this water could be coextracted with the SF, and reach the enzyme-bed. Therefore, when using enzymes in automated analytical SFE system, it is important to dry the sample before extraction, in order to control the water content subsequently of the immobilized enzyme. Snyder et al. (37, 78) showed that removal of water from meat and oilseed samples prior to SFE was critical for a successful extraction/lipase-catalyzed reaction of lipid matrices. Drying of the sample can be achieved by air-drying, freeze-drying or mixing with a drying agent such as Hydromatrix®, basic alumina, molecular sieves or magnesium sulfate. In an analytical study by Snyder et al. (78); freeze-drying was found to be more efficient than Hydromatrix®. However, both drying methods gave higher yields of FAMEs for the CALB-catalyzed methanolysis reaction of triacylglycerols extracted from meat and oilseed samples, compared to the untreated samples.

Analysis procedures

The extent of the reaction can be continuously monitored by visual examination if the reaction cell is equipped with a sapphire window (12, 107), or by employing either an on-line coupled detector such as UV equipped with a high pressure flow-through cell (74) or alternatively, with an on-line chromatograph such as HPLC (52). It has also been demonstrated by Berg et al. (109) that an analytical SFE/SFR system can be coupled on-line with a SFC employing FID detection, in the determination of methyl- and butyl

fatty acid esters. King and coworkers have further demonstrated the efficacy of automatic extraction/reaction/determination procedure for the determination of FAMEs using on-line SFE/SFR-GC (37, 69).

However, off-line SFE/SFR methodologies are most commonly used, in which lipid compounds are determined by employing HPLC with UV (101), ELSD (97), or RI (110) detection, GC-FID (38, 91), or SFC-FID (69, 105). The selectivity of the SFE/SFR method can be improved by employing fractionated collection, particularly if available on an automated SFE system. This was substantiated by the study by Marty et al. (111), in which a continuous reactor with four separators for collection enabled good separation of a produced ester from unreacted oleic acid.

In analytical studies, the calculated yield of the SFE/SFR process is usually based on the "true" or expected content of the analyte in the sample. If spiked samples are used, the analyte content is intrinsically known. However, the use of spiked samples does not give a true picture of the SFE/SFR procedure, since the extraction of naturally-occurring analytes from a real sample is usually much slower than the extraction of analytes, spiked in the same sample. If real samples are used, the analyte content has to be defined as accurately as possible. This can be done employing conventional methodology and setting the "100% recovery" based on an average of these experimental results. An alternative approach is to send the samples to a referee laboratory for analytical determination. Another approach is to use a standard reference material (SRM) in which the analyte content has been thoroughly determined by several laboratories employing different methodologies. However, if the objective is to evaluate a SF-based method, it can be included in an arranged collaborative study, in which a large number of laboratories participate (112).

Applications of enzymes with critical fluids

Enzymes have frequently been used in SFs for synthesis of high-value lipids (30, 34, 113, 114). Examples include RML-catalyzed hydrolysis of canola oil for the production of mono- and diacylglycerols, free fatty acids and glycerol (105), the CALB-catalyzed alcoholysis of cod liver oil for the production of ethyl esters of long-chain n-3 polyunsaturated fatty acids (97), the CRL-catalyzed esterification of fatty acids from milk fat for the formation of flavor esters (91), or the RML-catalyzed interesterification of palm oil and tristearin for the synthesis of cocoa butter equivalents (115). Analytical applications have been mainly concerned with quantitative extraction/lipase-catalyzed alcoholysis of oleaginous materials in-situ for the production of FAMEs, directly followed by off or on-line GC analysis. However, lipases have also been used in analytical SFE/SFR for hydrolysis and alcoholysis reactions for the determination of fat-soluble vitamins in foods, thereby avoiding the need for sample clean-up prior to HPLC analysis. These different analytical applications will be discussed in more detail below.

Fat determination for food labeling purposes today has to follow the definition established by the Nutritional Labeling and Education Act (NLEA), i.e. nutritional fat is a sum of fatty acids from mono-, di- and triacylglycerols, free fatty acid, phospholipid fatty acids and sterol fatty acids, etc (116). The conventional methodology for fat determination recommended by NLEA also includes acid- or alkaline-hydrolysis, solvent extraction, followed by chemical derivatization reaction for the production of FAMEs with final analysis by GC. This methodology is tedious and consumes large amounts of organic solvents. An automated SFE/SFR method in many aspects would be a welcomed alternative.

In an early study by Berg et al. (109), an SFE unit was coupled on-line to a capillary SFC employing MS or FID detection. The reaction catalyzed by an immobilized RML was alcoholysis of edible fat to methyl and butyl fatty acid esters. The extraction cell was loaded with segmented layers from the inlet side to the outlet side with (i) hydrated silica (to provide the enzyme with water); (ii) cotton wool with the alcoholic substrate; (iii) a mixture of edible fat, lipase and phosphate buffer (pH 7); (iv) cotton wool; and (v) dried Na_2SO_4 (to remove excessive water that could cause plugging of the restrictor with ice). Supercritical carbon dioxide at a pressure of 150 bar (2176 psi) and a temperature of 50°C was thus used to conduct the SFE/SFR. However, recovery data with respect to the formed methyl and butyl fatty acid esters were not given.

Jackson and King (100) demonstrated that when corn or soybean oil and methanol were simultaneously pumped into SC-CO_2, and then passed through an enzyme-bed of immobilized CALB, that high yields of resultant FAMEs could be obtained. Two Isco 100 DX syringe pumps were used for delivering SC-CO_2 and another two for the above reactants, resulting in the quantitative formation of FAMEs as determined by capillary GC-FID. These extraction/lipase-catalyzed reactions were performed at 24.1 MPa and 50°C. The obtained extraction recoveries were in the range 85-95%, and the product contained >95% FAMEs. The effect of methanol flow rate and water content on the conversion of triacylglycerols to FAMEs were investigated, and indicated that a methanol flow rate of 5 µL/min and dry SC-CO_2 gave optimal results. These promising results served as a basis for further analytical studies.

In an analytical study, Snyder et al. (37), determined the total nutritional fat content in meat samples by employing SFE/SFR via CALB-catalyzed alcoholysis and in-line determination of the resultant FAMEs by GC/FID. The interface between the SFE and

the GC instruments was a Hewlett Packard designed "bridge" system, where a robotic arm transferred the analytical vials from the SFE collection tray to the GC autosampler tray. This fully automated system is shown in Figure 5.

Figure 5

The extraction cells were loaded similarly as described previously (109), and included one layer consisted of a homogenized, freeze-dried meat sample and another layer containing the immobilized lipase: both layers separated by glass wool in the extraction cell. In this study, it was shown that the immobilized CALB catalyst could be washed and reused at least 25 times without a decrease in activity. SFE/SFR conditions of 12.16 MPa and 50°C using a SC-CO_2 flow rate of 1 mL/min, and a methanol flow rate of 5 µL/min, dynamic extraction time of 30 min, yielded 99.5% or better conversion of the extracted triacylglycerols to FAMEs. In addition, no significant differences were found between results using the described method on two different instruments: a Hewlett-Packard 7680T extractor coupled in-line to a GC or an off-line Isco system utilizing three Isco 100DX pumps. These results also did not differ significantly from results obtained using conventional methodology based on solvent extraction and BF_3-catalyzed esterification of the same samples. However, gravimetric determination of the total fat in these samples by conventional methodology gave significantly higher values, most likely due to coextraction of water and other extraneous, non-lipid components.

The effect of moisture in the sample on both lipid extraction and lipase-catalyzed alcoholysis via SFE was also studied (78). Using a similar method and the same SFE/SFR system (37), additional samples (meat samples, oilseeds of canola, soybean, sunflower and wheat germ) were analyzed for lipid content. These samples were either mixed with Hydromatrix®, freeze-dried or not pre-treated at all. The results showed that

water in the sample inhibits the extraction of lipids and the CALB-catalyzed conversion of triacylglycerols to FAMEs. Freeze-drying of the samples turned out to be the most efficient sample pre-treatment, yielding significantly different results from those obtained from mixing with Hydromatrix®. In this study, it was also demonstrated that cholesteryl esters could be quantitatively extracted and converted to cholesterol and FAMEs, as could phospholipids, yielding enzymatic conversion >96% and analyte recoveries ranging from 88 to 99%. The determined total fat/oil content in these meat and oilseed samples were similar to those obtained by doing Soxhlet extraction on the same samples.

In an interesting analytical application reported by King et al. (38), the total fatty acid content of soapstocks derived from soybeans and corn oil processing was determined. A similar SFE/SFR methodology as described above (37) was used, with immobilized CALB as the catalyst. Both Hewlett-Packard 7680T and Isco SFX-3560 SFE instruments were utilized to demonstrate the technique. FAME analysis of the extracts (products) from both extractions was done by capillary GC-FID. This methodology was then compared to an official AOCS method (G3-53) based on saponification followed by solvent extraction and gravimetric determination as well as a simple SFC method. The protocols of these three methodologies are shown in Figure 6.

Figure 6

The AOCS method yielded slightly lower values of FFA content of the soapstocks than the SFE/SFR method, but higher than a corresponding rapid SFC method that was also reported. In addition, the SFE/SFR method was fully automated, faster (3 h, in contrast to 5-8 h required by the AOCS method) and used considerably less organic solvent (1.8 mL, compared to 575 mL using the AOCS method). However, as the author pointed

out, SFC is a simple and fast alternative technique (only ¾ h), which yields an approximate determination of the FFA level in soapstocks (using only 8 mL of solvent per sample), and thus could be used for some applications.

In an analytical application by Turner and McKeon (39), the content of cis-vaccenic acid in milkweed seeds was determined using immobilized CALB in SC-CO_2 for extraction and methanolysis of seed oil to FAMEs. Two different types of carrier materials for the enzyme were tested -a hydrophobic polymer (Novozyme 435) and a silica-based material (NovoSample 40013), both from Novozymes A/S. Several SFE parameters were investigated to find the optimal conditions, including extraction time, temperature, methanol concentration, and water level. The optimal methanol and water concentrations differed for the two enzyme preparations studied, in that NovoSample 40013 was more sensitive to methanol (the lowest concentration tested, 1%, gave the highest yield, while 3% was optimal for Novozyme 435), and NovoSample 40013 required a small amount of water to be active (0.03%) while Novozyme 435 gave the highest yield at zero water concentration. Overall, Novozyme 435 demonstrated the fastest reaction kinetics and consistently gave the highest cis-vaccenate yields. The optimized methodology was applied on 15 species of milkweed seeds, giving an average recovery of 105 +/- 7% when compared to results obtained using a conventional methodology.

In another analytical study by Turner et al. (40), Novozyme 435 was used in an Isco 3560 extraction system for analysis of castor oil. Statistical design was used to find the optimal experimental conditions from varying pressure, temperature, methanol concentration and water concentration. Response surfaces were plotted, and these showed trends leading to nearly 100% yield for both the extraction and transmethylation

of the castor oil. The optimal parameters included the use of SC-CO_2 at 300 bar (4351 psi) and 80°C with 7 vol% methanol concentration and 0.02 vol% water concentration. These conditions were then used for the determination of oil content in castor seeds expressed as FAMEs. The results obtained using this enzyme-based methodology were similar to those obtained using conventional solvent-extraction/chemical transmethylation.

In a non-analytical study, but using analytical scale extraction/reaction equipment, King et al. (117) investigated the synthesis of sterol esters from their component fatty acids and sterols in SC-CO2. Four enzymes: Novozyme 435, Chirazyme L-1, Chiarzyme L-3, and Lypozyme IM were individually screened for their synthetic utility, Chirazyme L-1 proving to be the optimal lipase for above synthesis. A ladder of pressures (20.7 - 31 MPa), temperatures (40-60°C), fatty acid chain lengths (C8-C18), were utilized to characterize the feasibility of enzymatic-catalyzed synthesis. Variables such as flow rate and static hold times, as well as a scheme for the continuous addition of reactants were also studied. High yields of the corresponding sterol esters were achieved using two model sterols: cholesterol and sitostanol, ranging from 90-99%. Slightly higher conversion of the corresponding sterol esters was found as the chain length of reacting fatty acid increased. The described method appears to be a facile way of synthesizing cholesterol-reducing sterol esters, but could also have utility in preparing specific ester derivatives for analytical purposes.

Some lipases can be used to catalyze the hydrolysis/alcoholysis of fat-soluble vitamin esters. In two studies by Turner et al. (42, 76), it was shown that vitamin A (retinol) and vitamin E (tocopherols) could quantitatively be determined in various food items using immobilized CALB to hydrolyze both retinyl esters, thereby facilitating final analysis,

and integrated sample clean-up. The extraction cell was loaded as previously described (37); one layer of homogenized sample mixed with Hydromatrix® (inlet side), one layer of immobilized enzyme (outlet side), both layers being separated by glass wool. Ethanol was added both initially to the sample and continuously to the SC-CO_2. Samples were analyzed by RP-HPLC using UV and fluorescence detection, off-line after SFE/SFR. In the first study (42), the effect of using different lipases, pressures, temperatures, extraction time and modifier concentrations was investigated for the determination of vitamin A in milk powder. The optimized SFE/SFR methodology was tested on infant formula (NIST Standard Reference Material 1846) minced pork- and beef meat and low- and high-fat liver paste, giving good recoveries of vitamin A and E when compared to methodologies based on alkaline hydrolysis (saponification).

In the second study (76), six lipases and one esterase were screened for activity with retinyl palmitate and α-tocopheryl acetate in solvent systems having different water activity (a_w): (i) high a_w in water-saturated di-isopropyl ether (~1.2%, w:w); (ii) low a_w in n-hexane/ethanol [87.5/12.5 (v:v)]; and (iii) medium a_w in SC-CO_2/ethanol/water [97/2.85/0.15 (v:v:v)] mixture. Three lipases, CALB, RML and PCL, consistently gave the highest conversion of retinyl palmitate to retinol for all the solvent systems tested. None of the enzymes showed any activity towards α-tocopheryl acetate, presumably due to steric hindrance of this analyte at the enzyme's active site. Immobilized CALB showed the highest activity and stability at supercritical conditions, and a larger tolerance to different water activities and higher temperatures. The optimal SFE/SFR parameters using SC-CO_2 containing 3 vol% ethanol and 0.03 vol% water, were a pressure of 366 bar (5308 psi) and a temperature of 80°C (density of 0.8 g/mL), a 5 min static extraction followed by 75 min dynamic extraction at 0.5 mL/min, followed by a

45 min dynamic extraction at 1.0 mL/min, to assure quantitative extraction of the vitamins and to avoid breakthrough losses of unreacted vitamin esters. This methodology gave 100% recovery or higher of vitamin A and E in milk powder and infant formula, when compared to results obtained with the same samples employing traditional methodologies based on saponification. This would confirm that saponification is a harsh treatment which can lead to degradation of the sensitive fat-soluble vitamins (118), and overall supports that enzymatic hydrolysis is a superior sample preparation method for accurate determination of the vitamins in foodstuffs.

Taylor and King (41) explored the possibility of applying a SFE/SFR scheme for the analysis of both fatty and resin acids in tall oil products. Six different lipases were screened for synthetic efficacy: Novozyme SP 435, Lipozyme IM, Chirazyme L-1, as well as three laboratory prepared, supported lipases on Accurel EP100. Using an automated Isco Model 3560 extractor and the previously described segmented bed approach in the extraction cell (37), the first three of the above enzymes were evaluated for their ability to form FAMEs as well as resin acid methyl esters (RAMES). The Accurel-supported lipases were similarly evaluated using an Isco SFX-2-10 extractor SFE system due to the more limited quantities of these supported enzymes. Using the SFE/SFR conditions previously reported by Snyder et al. (37), it was found none of the above Accurel-supported enzymes produced either FAMEs or RAMES. However, Novozyme SP 435, Lipozyme IM, and Chirazyme L-1, produced FAMEs and to varying degrees, RAMES; the best results as noted previously being attained using Novozyme SP 435. However even Novozyme SP 435 gave only qualitative conversion of tall oil to the component RAMES. Synthesis of the methyl esters of tall oil resin acids for analytical purposes is difficult even by conventional derivatization methods, due

largely in part to bulky substituents which impede reaction of the methanol with the carboxylic acid group of the resin acid. However the reported SFE/SFR method does provide an analytical method for differentiating between the two types of acid moieties in tall oil mixtures.

In an innovative study by Bauza et al. (49), immobilized horse radish peroxidase (HRP) and cholesterol oxidase (COD) were used in SC-CO_2 for analysis of hydrogen peroxidase in vegetable oils and cholesterol in fat samples like whole milk, egg yolk, chicken and beef fat, lard and fish liver oil. The principle of the reaction is that cholesterol extracted from the sample reacts with oxygen to form 4-cholesten-3-one and hydrogen peroxide, a reaction catalyzed by COD. The hydrogen peroxide produced reacts with 4-amino antipyrine and 2-chlorophenol to form a quinoid dye, a reaction catalyzed by HRP. The quinoid dye strongly absorbs light at 554 nm, and analysis was performed by HPLC. The enzymes were immobilized on controlled pore glass beads, and demonstrated good stability (~16 hours of use) in SC-CO_2 containing 5% toluene saturated with phosphate buffer containing 1% ethanol.

Conclusions and further recommendations

In the above review, we have documented the use of enzymes with supercritical fluid media, namely SC-CO_2, to facilitate sample extraction, preparation, and analysis. As noted in the numerous cited applications, inclusion of enzymes in SF-based methods can facilitate an integrated scheme incorporating the above three discrete analysis steps. To date the use of lipases for facilitating transesterification or hydrolysis seems particularly promising when applied to the analysis of lipid-type analytes that exhibit high solubilities in SC-CO_2. Such enzymes could also be incorporated in additional

applications and even with polar analytes whose solubility may be enhanced by the addition of either a non-reactive or reactive cosolvent to SC-CO_2. It should be stressed that SF conditions which may work with one enzyme may not be appropriate for another type of enzyme, however one of the advantages of SF-based analytical methods is that conditions or fluids can be readily altered to be compatible with the catalytic activity of many enzymes. The use of mixtures of enzymes, e.g., mixed lipases has hardily been explored and this could expand the use of SFE/SFR-based techniques and make them more rigorous.

The preparation of samples off-line before enacting SFE has been documented in the literature (119) and non-enzymatic SFE/SFR schemes have been documented numerous times in the literature on a plethora of applications (35, 120-122). Certainly the ability to extract and derivatize simultaneously in an environmentally-responsible manner makes SFE/SFR-based methods attractive to the analytical chemist. Enzymatic action on the sample matrix to "free up" bound analytes has a precedent in the literature, but has only been sparingly applied in conjunction with analytical supercritical fluid techniques. For example, the application of carbohydrases may have merit in modifying the sample matrix in the presence of a supercritical fluid, or alternatively to facilitate a reaction with a target analyte. The recent application of analytical subcritical water extraction to biological matrices (123) could provide the proper medium and conditions for exploiting this particular class of enzymes.

The transesterification studies in SC-CO_2 to form FAMEs as reported in this review have other interesting applications. For example, these analytical-scale derivatization can be used to model SFR for potential scale-up to an engineering level for the "green" processing of a number of industrially-useful products. Present incidents of microbial

food contamination and attack suggest potential applications of reported SF-derivatization methods for rapidly identifying microorganisms via their FAME profiles (124-126). The above techniques can readily be applied to small samples, such as portions of a seed (127) or insects, with or without derivatization of the analyte. There is much to be learned regarding other possibilities from reading the literature concerned with industrial or engineering applications of SFs (128-130) as well as the literature on ultra-high pressure food processing (131, 132), where control of enzymatic activity in foods is paramount to improving or producing longer lasting shelf life products.

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Figure captions

Figure 1 Published papers concerning the utilization of enzymes as catalysts in SFs, obtained from chemical abstract on-line.

Figure 2 Mechanism for a lipase-catalyzed reaction of triacylglycerol.

Figure 3 Retinol recoveries obtained by enzymatic reaction of retinyl palmitate using immobilized CALB and RML in analytical SFE (n=3). The water activities of the immobilized enzymes were set to 0.43 and the density was maintained at 0.8 g/mL by using different pressures for the temperatures investigated. Reference taken from (76).

Figure 4 Schematics of different types of reactor modules used in SF applications [partly redrawn from ref (27)]. Abbreviations: SF=supercritical fluid, S=substrates, E=enzyme, P=products and C=chromatograph.

Figure 5 Fully automated SFE/SFR/GC instrument [redrawn from ref. (37)].

Figure 6 Comparison of the AOCS official method G3-53 for fatty acid content of soapstock with SFE/SFR and SFC methods [redrawn from ref. (38)].

Tables

Table 1 Abbreviations (Abbr.), selectivity (in organic solvents) and molecular weights (MW) of some lipases that have been used as catalysts in SFs, taken from reference (60). Representative references from the literature are shown in the rightmost column.

Lipase	Lipase family	Abbr.	Selectivity	MW	SF ref.
Porcine pancreatic	Mammalian	PPL	1,3-selective	50 kDa	(12)
Candida rugosa	Fungal *Candida rugosa*	CRL	nonselective	60–65 kDa	(133)
Rhizomucor miehei	Fungal *Rhizomucor*	RML	1,3-selective	30–35 kDa	(134)
Rhizopus oryzae	″	ROL	1,3-selective	″	(66)
Humicola lanuginosa	″	HLL	~nonselective	″	(135)
Penicillium camembertii	″	PcamL	1,3-selective	″	(115)
Candida antarctica B	″	CALB	1,3-selective	″	(136)
Aspergillus niger	Fungal Unclassified	ANL	1,3-selective	-	(137)
Pseudomonas cepacia	Bacterial *Pseudomonas*	PCL	nonselective	30–35 kDa	(74)
Pseudomonas fluorescence	“	PFL	nonselective	“	(138)
Bacillus thermocatenulatus	Bacterial *Staphylococcus*	BTL2	-	40–45 kDa	(137)

Figures

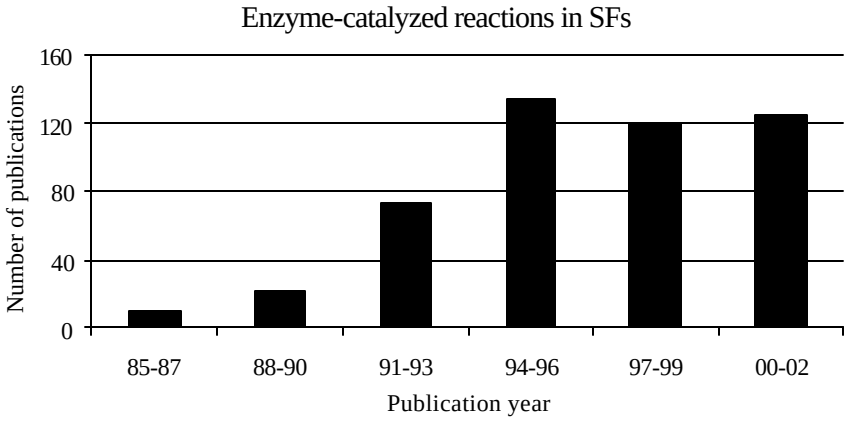
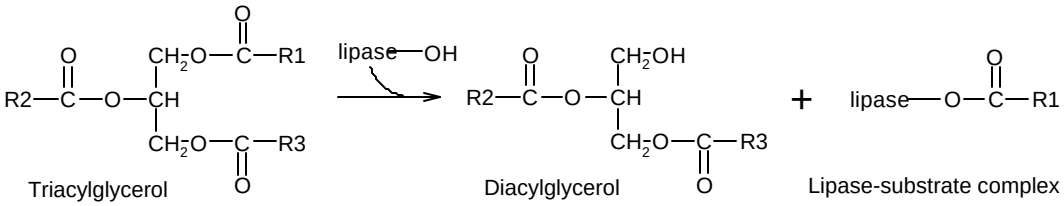


Figure 1

Step 1:



Step 2:

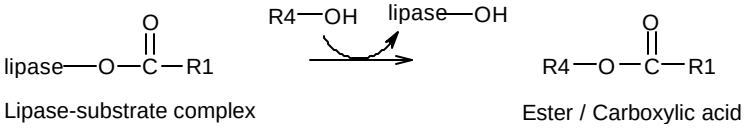


Figure 2

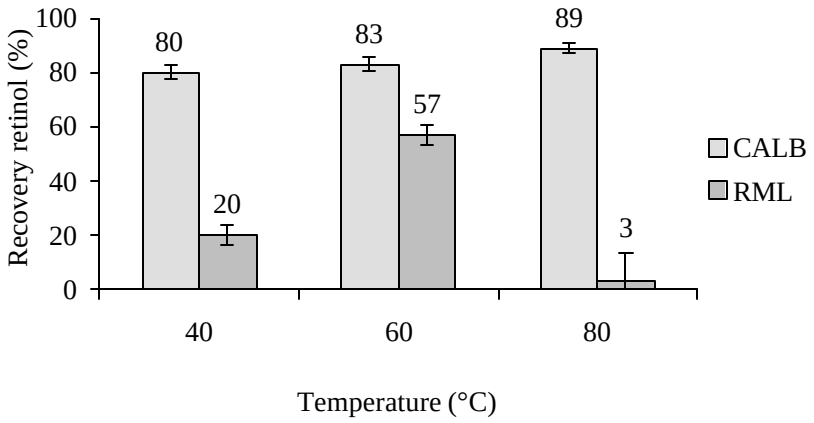
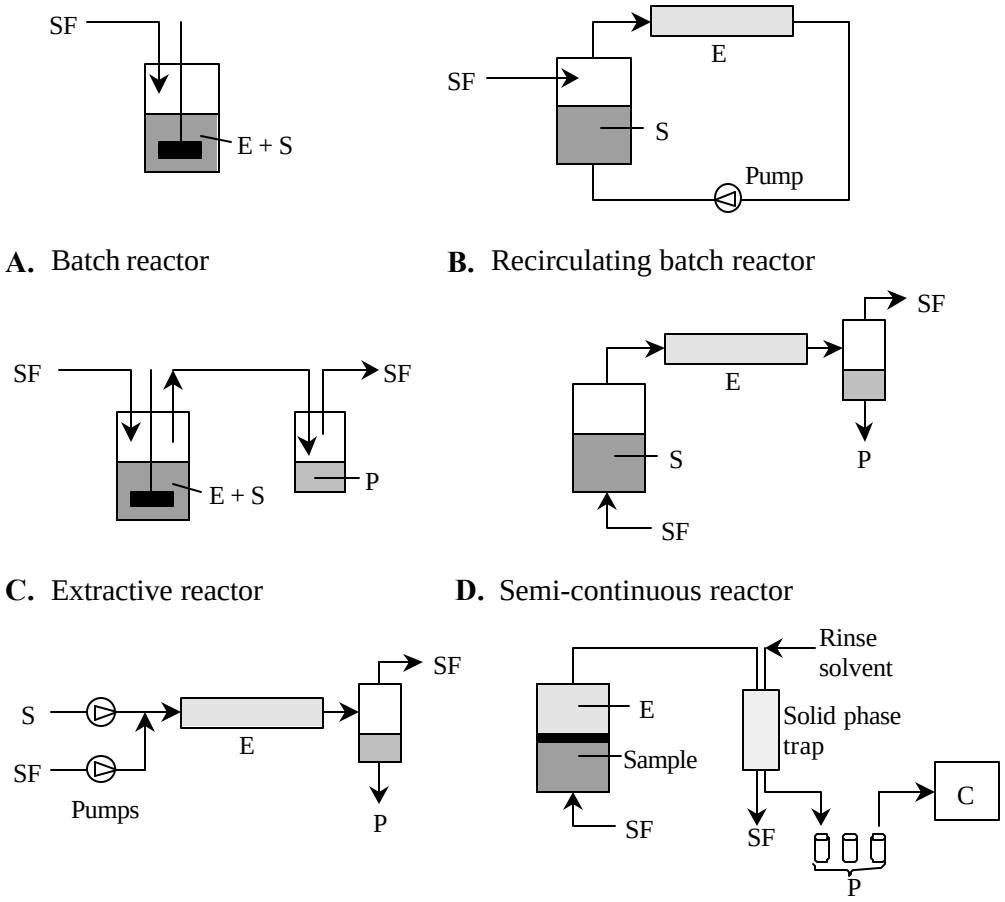

Recovery retinol (%)
100
80
60
40
20
0
80
20
83
57
89
3
40
60
80
Temperature (°C)
CALB
RML

Figure 3



A. Batch reactor

B. Recirculating batch reactor

C. Extractive reactor

D. Semi-continuous reactor

E. Continuous reactor

F. Analytical SFE/SFR system

Figure 4

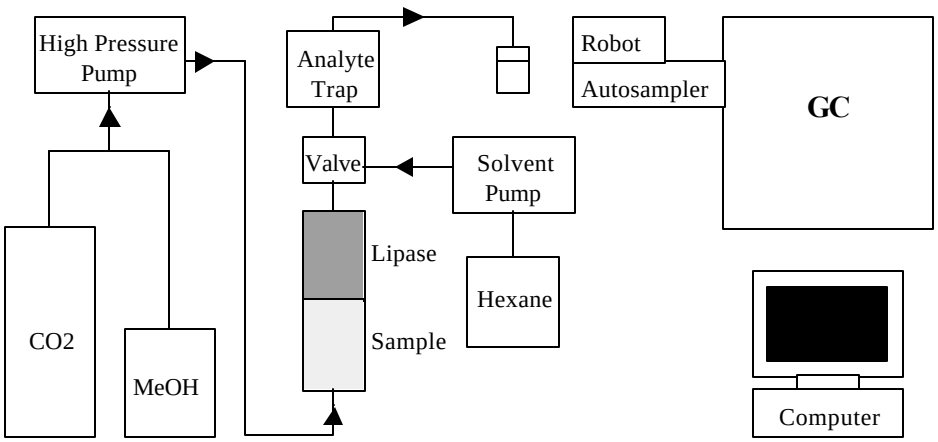

High Pressure Pump
Analyte Trap
Robot
Autosampler
GC
Valve
Solvent Pump
Lipase
Hexane
Sample
CO2
MeOH
Computer

Figure 5

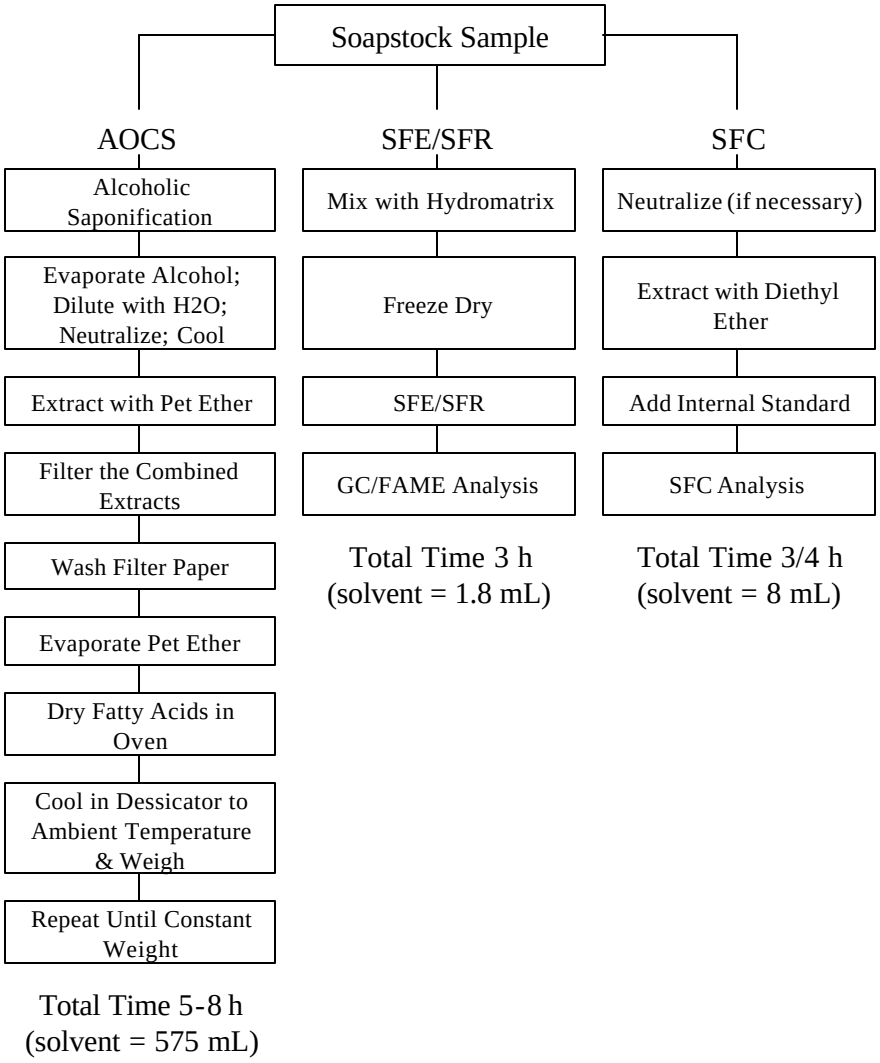

Soapstock Sample
AOCS
SFE/SFR
SFC
Alcoholic Saponification
Evaporate Alcohol; Dilute with H2O; Neutralize; Cool
Extract with Pet Ether
Filter the Combined Extracts
Wash Filter Paper
Evaporate Pet Ether
Dry Fatty Acids in Oven
Cool in Dessicator to Ambient Temperature & Weigh
Repeat Until Constant Weight
Total Time 5-8 h
(solvent = 575 mL)
Mix with Hydromatrix
Freeze Dry
SFE/SFR
GC/FAME Analysis
Total Time 3 h
(solvent = 1.8 mL)
Neutralize (if necessary)
Extract with Diethyl Ether
Add Internal Standard
SFC Analysis
Total Time 3/4 h
(solvent = 8 mL)

Figure 6